

(19) World Intellectual Property
Organization
International Bureau



03 MAR 2005

(43) International Publication Date
18 March 2004 (18.03.2004)

PCT

(10) International Publication Number
WO 2004/022039 A2

(51) International Patent Classification⁷: **A61K 31/00**

(21) International Application Number:
PCT/CA2003/001291

(22) International Filing Date:
3 September 2003 (03.09.2003)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/407,310 3 September 2002 (03.09.2002) US

(71) Applicant (for all designated States except US): **NEUROLOGICAL TECHNOLOGIES INC.** [CA/CA]; 120 Newkirk Road, Unit #2, Richmond Hill, Ontario L4C 9S7 (CA).

(72) Inventors; and

(75) Inventors/Applicants (for US only): **CACIAGLI, Francesco** [IT/IT]; Via Dei Vestini, 172-C, I-66013 Chieti (IT). **CICCARELLI, Renata** [IT/IT]; Via Dei Sabelli, N. 40, I-66100 Chieti (IT). **DI IORIO, Patrizia** [IT/IT]; Via Dei Vestini, 172/C, I-66013 Chieti (IT). **KLEY-WEGT, Sonya** [CA/CA]; 31 Carrington Place, Guelph, Ontario N1G 5C2 (CA). **WERSTIUK, Eva, Susanne**

[CA/CA]; 38 Whitton Road, Hamilton, Ontario L8S 4C7 (CA). **RATHBONE, Michel, P.** [CA/CA]; 15 Ricardo Court, Hamilton, Ontario L8W 2S1 (CA). **VERTES, Eva** [CA/CA]; 5 Highland Park Drive, Dundas, Ontario L9H 3L7 (CA).

(74) Agent: **BERESKIN & PARR**; 40 King Street West, 40th Floor, Toronto, Ontario M5H 3Y2 (CA).

(81) Designated States (*national*): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

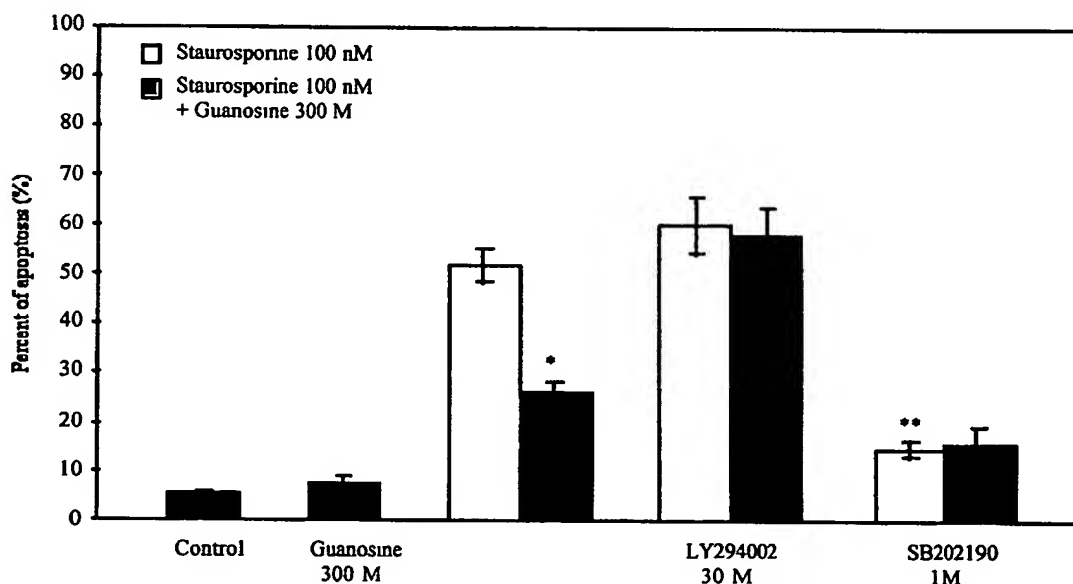
(84) Designated States (*regional*): ARIPO patent (GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PT, RO, SE, SI, SK, TR), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

[Continued on next page]

(54) Title: PURINE NUCLEOSIDES AS ANTI-APOPTOTIC AGENTS



(57) Abstract: Methods and compositions for preventing apoptosis are described. The method involves administering an effective amount of a purine nucleoside to a cell or an animal in need thereof. The methods are useful in treating diseases of the nervous system including neurodegenerative diseases, stroke or spinal cord injuries.

BEST AVAILABLE COPY

WO 2004/022039 A2



For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

TITLE: PURINE NUCLEOSIDES AS ANTI-APOPTOTIC AGENTS**FIELD OF THE INVENTION**

The invention relates to methods and compositions for preventing apoptosis.

5 BACKGROUND OF THE INVENTION

Apoptosis, or programmed cell death, is a gene-mediated mechanism associated with normal physiology and biological processes like proliferation and differentiation. Apoptosis plays an important role in normal neuronal development and in acute chronic pathological processes such as trauma, stroke, Huntington's chorea, prion diseases such as Creutzfeldt-Jacob disease, scrapie and bovine spongiform encephalopathy, and Alzheimer's disease (McConkey and Orrenius, 1994). How particular types of cells are targeted or protected is accomplished by activating genes that encode products such as caspases (cysteine-aspartate proteases) to commit cell suicide or proteins of the Bcl-2 family of proteins to protect them.

Apoptosis can be divided essentially into three phases; initiation, effector and a final outcome of cell death. The initiation phase may be activated by either an external (TNF receptor, Fas ligand) or an internal signal from the cell (oxidative stress or excess Ca^{2+} release) to activate specific signal transduction pathways. After a signal has been initiated, "check points" mediate the effector stage such as, gene products of the Bcl-2 related family of proteins and/ or cysteine aspartate proteases. It is the relative abundance of pro-apoptotic and/or the anti-apoptotic signals that will determine if a cell will be committed to undergo apoptosis. The final outcome of apoptosis is fragmented apoptotic bodies, which are eliminated by phagocytic cells without an inflammatory response. It is important to recognize that apoptosis is not a single process, but rather several process. Thus an intervention that prevents apoptosis in one condition may have no effect on apoptosis in other conditions.

30 Purines, in the nervous system (central and peripheral) can act as neurotransmitters and mediate trophic effects. These effects may lead to changes in cell metabolism, proliferation, and/ or release of certain growth

-2 -

factors. Purine nucleosides (adenosine, guanosine, inosine) are known to have many neuroprotective and neurorestorative effects in the peripheral and central nervous system.

SUMMARY OF THE INVENTION

5 The present inventors have demonstrated that purines nucleosides such as guanosine and inosine are capable of exerting an anti-apoptotic signal in cells of the central nervous system. The inventors have also shown that guanosine acts through a PI 3-K/ Akt (protein kinase B) pathway that is capable of protecting cells from further undergoing the process of apoptosis.

10 Accordingly, the present invention provides a method for preventing apoptosis in a cell of the nervous system comprising administering an effective amount of a purine nucleoside to a cell or animal in need thereof.

 The present invention also includes a pharmaceutical composition for use in preventing apoptosis comprising an effective amount of a purine
15 nucleoside in admixture with a suitable diluent or carrier.

 Other features and advantages of the present invention will become apparent from the following detailed description. It should be understood, however, that the detailed description and the specific examples while indicating preferred embodiments of the invention are given by way of
20 illustration only, since various changes and modifications within the spirit and scope of the invention will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

 The invention will now be described in relation to the drawings in
25 which:

 Figure 1 is a bar graph showing the effect of guanosine on staurosporine-induced apoptosis and intracellular pathways.

 Figures 2A and B show guanosine-mediated phosphorylation of Akt (protein kinase B) in cultured rat astrocytes as evaluated by Western blot
30 analysis.

 Figure 3A-C shows the effect of guanosine on phosphorylation of Akt/PKB in cultured rat astrocytes.

-3 -

Figure 4 demonstrates that guanosine promotes phosphorylation of glycogen synthase kinase-3 β (GSK-3 β).

Figure 5 demonstrates that guanosine induces the expression of Bcl-2 mRNA and protein in rat cultured astrocytes.

5 Figure 6 is a bar graph showing the effect of propentofylline, pertussis toxin, and antagonists of adenine-base purines on the protective effect of guanosine against staurosporine-induced apoptosis in cultured astrocytes.

DETAILED DESCRIPTION OF THE INVENTION

As hereinbefore mentioned, the present inventors have demonstrated
10 that the purine nucleosides guanosine and inosine are capable of preventing apoptosis in cells of the nervous system, such as neurons and astrocytes. The inventors have shown that guanosine can protect against apoptotic cell death caused by a number of known inducers or stimulators of apoptosis. In one example, the inventors have shown that guanosine can protect astrocytes
15 from apoptosis induced by staurosporine which is a potent inducer of apoptosis. In another example, the inventors have shown that guanosine can protect neuronal cells from apoptosis induced by β -amyloid protein which is known to induce apoptosis of neurons in Alzheimer's disease. These results demonstrate the applicability of the present invention to treating Alzheimer's
20 disease. In a further example, the inventors have demonstrated that guanosine protected astrocytes and neuroblastoma cells from apoptosis induced by combined hypoxia-hypoglycemia using combined oxygen-glucose deprivation (CGOD). As CGOD is an *in vitro* model of cerebral ischemia, the results demonstrate the applicability of the method of the invention in treating
25 conditions involving cerebral ischemia such as stroke or cerebral hypoxia due to circulatory insufficiency.

Accordingly, the present invention provides a method of preventing apoptosis in a cell of the nervous system comprising administering an effective amount of a purine nucleoside to a cell or animal in need thereof.
30 The present invention also provides a use of an effective amount of a purine nucleoside for the manufacture of a medicament for preventing apoptosis in a cell of the central nervous system.

The term "effective amount" as used herein means an amount effective, at dosages and for periods of time necessary to achieve the desired result (e.g. preventing apoptosis).

The term "animal" as used herein includes all members of the animal
5 kingdom, including humans. Preferably, the animal to be treated is a human.

The term "a cell" as used herein includes a single cell as well as a plurality or population of cells. Administering a purine nucleoside to a cell includes both *in vitro* and *in vivo* administrations.

The term "a cell of the nervous system" includes cells from both the
10 peripheral and central nervous system. Preferably the cell is from the central nervous system, most preferably the cell is an astrocyte or a neuron.

The term "preventing apoptosis" means that the level of apoptosis in the presence of the purine nucleoside is decreased or reduced as compared to the level of apoptosis observed in the absence of the purine nucleoside.
15 Apoptosis can be measured using a variety of known techniques, including measuring the activity of caspases, assessing DNA fragmentation (e.g. using the TUNEL assay described in Example 1) or by assessing cell viability (e.g. using staining method described in Example 1).

The inventors have shown that guanosine exerts its anti-apoptotic
20 effect through a PI 3-kinase/Akt/protein kinase β pathway. In particular, they have shown that the anti-apoptotic effect of guanosine is mediated by inactivation of glycogen synthase kinase-3 β (GSK-3 β), a downstream target of the PI3K/Akt/PKB pathway. Accordingly, the present invention provides a method of inhibiting glycogen synthase kinase-3 β comprising administering an
25 effective amount of a purine nucleoside to a cell or animal in need thereof. The present invention also provides a use of an effective amount of a purine nucleoside for the manufacture of a medicament for inhibiting the activity of glycogen synthase kinase-3 β in a cell of the central nervous system.

The inventors have also shown that guanosine induces Bcl-2 which is a
30 known anti-apoptotic protein. Accordingly, the present invention provides a method of inducing the expression and/or activity of Bcl-2 in a cell of the nervous system comprising administering an effective amount of a purine

-5 -

nucleoside to a cell or animal in need thereof. The present invention also provides a use of an effective amount of a purine nucleoside for the manufacture of a medicament for inducing the expression and/or activity of Bcl-2 in a cell of the central nervous system.

- 5 The purine nucleoside used in the above methods is preferably selected from guanosine, inosine and analogs thereof. Examples of analogs are provided below.

Derivatives by modifying the 2-amino-NH₂ group of guanosine:

	2 – methyl -	2-CH ₃ -
10	2 – ethyl -	2 – C ₂ H ₅ –
	2 – ethylamino -	2- CH ₃ CH ₂ NH –
	N,N – dimethyl -	2 – (CH ₃) ₂ N –
	2 – methylamino -	2 – CH ₃ NH –
	2 – ethylamino -	2 – CH ₃ CH ₂ NH –
15	N ₂ – benzoyl -	2 – C ₆ H ₅ C – NH –
	phenyl – amino -	2 – C ₆ H ₅ NH –

Substituting – phenyl – amino –

Derivatives by modifying the 6 – keto – C=O group of guanosine or inosine:

20	6 – thio -	6 – SH –
	6 – amino -	6 – NH ₂ –
	6 – chloro -	6 – Cl –
	6 - methoxy -	6 – OCH ₃ –
	6 - cyclopentyl -	6 – C ₅ H ₉ –
25	6 – cyclohexyl -	6 – C ₆ H ₁₁ –

Derivatives by substituting the imidazole ring of the purines:

	N7 – methyl -	7 – CH ₃ –
	C8 – hydroxy -	8 – OH –
	C8 – bromo -	8 – Br –

- 30 Derivatives by modifying the ribose ring of purine nucleosides:

2' – deoxy - ribose

2',3' – dideoxy – ribose

-6 -

5' – carbamido – derivatives

Changing the ribose to other sugars – eg: arabinose

Changing the ribose to carbocyclic analogues – eg: cyclopentane

Whether or not a particular analog of guanosine or inosine is useful in the methods of the invention can be tested using assays known in the art including the assays described herein. For example, the analog can be tested for its ability to prevent apoptosis using the assays described in the Examples. The analog can also be tested for its ability to inhibit GSK-3 β or to induce Bcl-2 using the assays described in the Examples.

10 The method of the invention can be used to treat any disease or condition of the nervous system wherein it is desirable to prevent apoptosis including, but not limited to, neurodegenerative diseases (including Alzheimer's disease, Huntington's chorea, Parkinson's disease, Bell's Palsy, Pick's disease, multiple sclerosis and amyotrophic lateral sclerosis), stroke, 15 head injuries, and spinal cord and other nerve crush injuries. The disease may also be a prion-induced disease such as Creutzfeldt-Jacob disease, scrapie, and bovine spongiform encephalopathy.

The present invention also includes pharmaceutical compositions containing purine nucleosides to prevent apoptosis in a cell of the central 20 nervous system. Accordingly, in one embodiment, the present invention provides a pharmaceutical composition for use in preventing apoptosis comprising an effective amount of a purine nucleoside in admixture with a suitable diluent or carrier. The purine nucleoside is preferably guanosine, inosine or analogs thereof.

25 The pharmaceutical compositions can be prepared by *per se* known methods for the preparation of pharmaceutically acceptable compositions which can be administered to patients, and such that an effective quantity of the active substance is combined in a mixture with a pharmaceutically acceptable vehicle. Suitable vehicles are described, for example, in 30 Remington's Pharmaceutical Sciences (Remington's Pharmaceutical Sciences, Mack Publishing Company, Easton, Pa., USA 1985).

- 7 -

On this basis, the pharmaceutical compositions include, albeit not exclusively, the active compound or substance in association with one or more pharmaceutically acceptable vehicles or diluents, and contained in buffered solutions with a suitable pH and iso-osmotic with the physiological fluids. The pharmaceutical compositions may additionally contain other agents such as other agents that can prevent the inhibition of apoptosis or that are used in treating inflammatory conditions or sepsis.

Such pharmaceutical compositions can be for intralesional, intravenous, topical, rectal, parenteral, local, inhalant or subcutaneous, intradermal, intramuscular, intrathecal, transperitoneal, oral, and intracerebral use. The composition can be in liquid, solid or semisolid form, for example pills, tablets, creams, gelatin capsules, capsules, suppositories, soft gelatin capsules, gels, membranes, tubelets, solutions or suspensions.

The pharmaceutical compositions of the invention can be intended for administration to humans or animals. Dosages to be administered depend on individual needs, on the desired effect and on the chosen route of administration.

The following non-limiting examples are illustrative of the present invention:

EXAMPLE

Example 1

MATERIALS AND METHODS

Cells

SK-N-SH culture

The SK-N-SH human neuroblastoma cells were purchased from ATCC and grown in Minimum Essential Medium (MEM) (Gibco, Burlington, ON) at 37°C in a humidified atmosphere of 95% air and 5% CO₂. MEM was supplemented with non-essential amino acids (0.1 mM), sodium pyruvate (0.1 mM), penicillin / streptomycin 100 U/ ml, and 10% fetal bovine serum (FBS) (Gibco, Burlington, ON). Cells were treated with 236 nM herbimycin A (Sigma, Mississauga, ON) and 10 µM retinoic acid (Sigma, Mississauga, ON) in order to differentiate the cells into a neuronal type (Preis et al., 1988). For

-8 -

individual experiments, SK-N-SH cells were harvested by washing cells one time with warmed 0.1 M phosphate buffered saline, and then treating the cells with 1 ml of trypsin-EDTA (0.05%, 0.53 nM) (Gibco BRL, Burlington, ON). Cells were centrifuged at 1000 rpm for 5 minutes. Cells were re-suspended in supplemented MEM and counted in the presence of Trypan blue (0.04%) using a hemocytometer to exclude non-viable cells. Cells were seeded at a concentration of 2.5×10^4 cells/well in a 24 well Falcon tissue culture plate and grown for 2 days. Cells were serum starved for 24 hours using supplemented MEM plus 0.5% FBS.

10 ***Astrocyte culture***

Primary cultures of rat astrocytes were prepared from cerebral cortex of 18-19 day-old-fetuses of Sprague Dawley rats as previously described (Diorio et al., 2002). Dissociated cortical cells were grown on poly-D-lysine-coated (10 µg/ml) T75 flasks using Dulbecco's Modified Eagle Medium (DMEM) culture medium containing 20% horse serum (HS) and 1% penicillin / streptomycin. Cultures were maintained in standard conditions, 37°C; 100% humidity; 95% air/5% CO₂. Initially astrocytes were treated with a lysomotrophic agent, L-Leucine methyl ester (5mM) (Sigma, Oakville, ON), to selectively destroy contaminating microglia (Giuliam and Baker, 1986). After which they were maintained in high glucose DMEM containing 5% HS and 1% penicillin / streptomycin with medium replacement every 3-4 days. After 12-14 days in vitro (DIV) the cells were agitated vigorously for 4 hours on an orbital shaker at 100 rpm to minimize microglial contamination (Giulian and Baker, 1986). Immunostaining for glial fibrillary acidic protein (Reinhart et al., 1990) showed that more than 97% of cultured cells were astrocytes, which had the typical appearance of type-1 astrocytes upon microscope examination. Astrocytes were detached from the culture flasks by treatment (5-10 min, 37°C) with 0.25% Trypsin/0.04% EDTA (GibcoBRL). Cells were counted in the presence of 0.04% Trypan Blue Stain (GibcoBRL) to exclude non-viable cells; this showed 97% viability. Astrocytes were resuspended in DMEM supplemented with 20% HS and 1% penicillin / streptomycin and seeded as follows: for the evaluation of apoptosis, cells were replated onto poly-D-lysine-

-9 -

coated round glass coverslips at cell concentration of 1×10^5 cells/coverslip; for Western blot and RT-PCR analyses astrocytes were replated onto poly-D-lysine-coated 100 mm dishes, respectively, at a cell concentration of 2×10^5 cells/dish. On the 2nd day after replating, media was replaced with DMEM
5 supplemented with 5% HS and 1% penicillin / streptomycin and bioassays were usually performed on the cultures 4 days after replating.

Treatment of cell cultures with various drugs

Astrocytes and SK-N-SH cells were treated with a variety of purines, at different concentrations as follows: guanosine 10-300 μ M or inosine 100 μ M.
10 In experiments, which evaluated apoptosis, astrocytes and/ or SK-N-SH cells were treated with 100 nM or 10 nM staurosporine, a potent inducer of apoptosis. Staurosporine is a potent inducer of apoptosis in neuronal and non-neuronal cells (Ahlemeyer et al., 2000; Koh et al., 1995; Mangoura and Dawson, 1998; Weisner and Dawson, 1996; Krohn et al., 1998, 1999). Cells
15 were treated with amyloid β -protein (25-35) at a final concentration of 5 μ M and 10 μ M. In experiments where cells were pre-treated with either purine nucleoside, the nucleoside was added one hour prior to the addition of either staurosporine or amyloid β -protein. In experiments to determine the effect of intercellular transduction pathways, astrocytes were treated with [4-(4-
20 fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole, FHPI] (SB202190), an inhibitor of the p38 mitogen-activated protein kinase pathway or [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] (LY294002) (Calbiochem), an inhibitor of phosphatidyl inositol 3- kinase or with adenosine receptor antagonists, DPCPX (100nM) or alloxazine (10 μ M) or P2 purine
25 receptor antagonists, suramin (10 μ M).

Certain experimental agents were dissolved in dimethylsulfoxide (DMSO) or sodium hydroxide (NaOH, 1N), all experiments were performed using solubility controls at a final concentration of 0.01% DMSO or 0.01% NaOH. All purines were purchased from Sigma unless otherwise stated.

30 Determination of apoptosis

a. Tunel assay

-10 -

DNA fragmentation was evaluated using the In Situ Cell Death Detection Kit: TUNEL assay [TdT(terminal deoxynucleotidyl transferase)-mediated dUTP nick end labeling] (Boehringer Mannheim) and performed according to the manufacturers instructions. Briefly, at the end of the treatments adherent astrocytes were fixed with 4% paraformaldehyde for 30 min at room temperature. Cells were washed twice with phosphate buffered saline and incubated in permeabilization solution (0.1% triton, 0.1% sodium citrate) for 20 minutes on ice (4°C) and assayed by TUNEL. Positive controls were included by incubating the fixed and permeabilized cells with DNase I (10 µg/ml) for 10 minutes at room temperature to induce DNA strand breaks. Finally the cells were examined by fluorescence microscopy.

b. Staining with acridine orange / ethidium bromide

SK-N-SH cells were stained for cell viability or apoptosis using acridine orange (AO) (3 µg/ml) or ethidium bromide (EB) (10 µg/ml) in 0.1 M phosphate buffer saline (PBS) solution. After treatment exposure, cells were rinsed twice with 0.1 M PBS and treated with AO/EB solution. Cells were visualized under fluorescence (485 nm). Live cells stained green and dead cells fluoresced orange/ red. Four individual fields were counted / well / treatment.

20 Western blot analyses

Phosphorylation of Akt / protein kinase B was evaluated by Western blot analysis. After 72 hours of re-plating astrocytes were serum starved in high glucose DMEM for 24 hours. Astrocytes were exposed to increasing concentrations of guanosine (30, 100 and 300µM) for 10 minutes at 37°C. In experiments using the inhibitor of PI 3-kinase, LY294002 (30 µM) was added to the culture medium 30 minutes prior to the addition of guanosine. At the end of the treatments, astrocytes were washed twice with ice-cold phosphate buffered saline (PBS) and harvested at 4°C in 25 mM Tris buffer pH 7.4 containing: 150 mM NaCl, 100 µM sodium orthovanadate, 1.5 mM MgCl₂, 1.0 mM EDTA, 1% NP40, 10% glycerol, 1 mM PMSF, 5 µg/ml leupeptin, 5 µg/ml aprotinin. Cells were disrupted by sonication and centrifuged at 14,000 rpm for 5 min at 4°C. Aliquots (20 µl) were removed from the supernatants for the

-11 -

determination of protein concentration. Samples were diluted in SDS-bromophenol blue buffer and boiled for 5 min before loading onto 12.5% SDS polyacrilamide gel, using 10 µg of total protein per lane. After separation, proteins were transferred to immuno-Blot PVDF membranes (Bio-Rad Laboratories). Membranes were incubated with an antibody against phosphorylated Akt [1:1000, rabbit polyclonal phospho-Akt (Ser473) antibody (New England Biolabs)] overnight at 4°C. Blots were then incubated for 1 hour with the secondary antibody (peroxidase-coupled anti-rabbit, 1:2000, Amersham). Immunostaining was revealed by the enhanced ECL western blotting analysis system (Amersham). Densitometric analysis was performed for quantification of the immunoblots. Assay of Caspase-3 Activity

The activity of caspase-3 was determined using a colorimetric protease assay kit (MBL, Nagoya, Japan). Cell lysates were prepared from 2×10^6 rat cultured astrocytes after various treatments. Aliquots of the lysates were diluted in 50 µl of chilled cell lysis buffer and incubated on ice for 10 min. After centrifugation at 10,000 x g for 1 min, the supernatants were stored on ice. A proteolytic reaction was carried out in a reaction buffer containing 50 µg of cytosolic protein extract and 200 µM of N-acetyl-Asp-Glu-Val-Asp-p-nitroanilide (Ac-DEVD-pNA). The reaction mixture was incubated at 37°C for 2 h and the formation of p-nitroanilide (pNA) was measured at 405 nm using a microtiter plate reader (Spectracount™, Packard Canberra, IL, USA). The activity of caspase-3, was calculated from the slope of absorbance over time, and is expressed as pmol of substrate cleaved/min/µg of cytosolic protein.

Identification of Bcl-2 mRNA by Northern Blot Analysis

Cultured rat astrocytes were pre-treated with guanosine (300 µM) for 4 hours, and total RNA was isolated from untreated and guanosine-treated cells using the TRIzol reagent (Life Technologies BRL, Milan, Italy) and quantified by a scanning spectrophotometer. Twenty micrograms of total RNA was electrophoresed on formaldehyde denaturing 1 % agarose gel and viewed under ultraviolet light (UV) to verify loading and the presence of intact ribosomal bands. The RNA was then transferred to a nylon membrane (Hybond-N, Amersham Pharmacia Biotech, Buckinghamshire, UK) and fixed

-12 -

by UV crosslinking (UV Stratalinker 1800, Stratagene). Bcl-2 cDNA probe was amplified by the polymerase chain reaction (PCR). Bcl-2 primers were designed based on a previously published rat cDNA sequences (Genbank accession number, RNBCL2A): Bcl-2 primer sense 5'-
5 CGGGACGCGAAGTGCTAT-3', Bcl-2 primer antisense 5'-
CCCAGCCTCCGTTATCCTGGA-3'. Bcl-2 insert was purified and radiolabeled by the method of random priming (Megaprime DNA labeling system, Amersham Pharmacia Biotech) using ³²P-labeled alpha-dCTP. Filters were prehybridized in a mixture containing 50% formamide, 1% sodium
10 dodecyl sulphate (SDS), 5 x tri-sodium citrate, sodium chloride (SSC), 5 x Denhardt's solution, and 100 mg/ml denatured salmon sperm. Hybridization was performed under the same conditions with the addition of labeled probe at 42°C overnight. Membranes were washed using 0.1% SDS/2 x SSC at room temperature and then twice in 0.2% SDS/1 x SSC at 50°C before being
15 exposed to Kodak Biomax MS film (Sigma). Subsequent to hybridization with Bcl-2 cDNA probe, filters were hybridized with a probe for GAPDH to allow correction for the recovered RNA in each sample. For the purpose of quantification autoradiograms were scanned using laser densitometry. Bcl-2 mRNA signals were normalized against GAPDH content by determining the
20 ratio of their respective optical densities.

RT-PCR

Effect of guanosine on the expression of Bcl-2 mRNA in rat cortical astrocytes was also determined by semi-quantitative RT-PCR. Total RNA was isolated from cultured rat astrocytes, untreated and treated with 150 µM
25 guanosine for 4 hours, and 2 µg were reverse-transcribed to cDNA. Standard curves were generated using serial dilutions of the RT reactions as templates for PCR with each primer set to determine the linear range of the assay. Products from the original RT reaction volumes were appropriately diluted into the final PCR volumes to ensure nonsaturation of the PCR amplification
30 reactions.

RESULTS

The anti-apoptotic effect of guanosine mediated by a G_i protein-coupled putative receptor for this nucleoside

The inventors have recently reported that specific binding sites exist for [³H]-guanosine in rat brain membranes (Traversa et al., 2002a), and these putative receptor sites are also expressed in cultured rat astrocytes (Traversa et al., 2002b). In the latter cells, as well as in whole brain membranes, guanosine binding is sensitive to treatment with pertussis-toxin (PTX), suggesting that the putative guanosine receptor is coupled to G_i proteins (Traversa et al., 2000; Traversa et al., 2002b). The inventors therefore investigated whether the anti-apoptotic effect of guanosine was mediated by these plasma membrane localized guanosine receptors. Astrocytes were exposed to 100nM staurosporine for 3 hours, and 300μM guanosine or vehicle (growth medium = control) was added 1 hour prior to the addition of staurosporine, and was present during the 3 hours of staurosporine treatment (total exposure time 4 hours). Cells were pre-treated with propentofylline (PPF, 100 μM), or the combined antagonists of adenine-base purines (ABP) (100 nM DPCPX + 30 μM DMPX + 10 μM suramin) for 30 min prior to the addition of guanosine. Pre-treatment with pertussis toxin (PTX, 200 ng/ml) was performed for 16 hours. The extent of apoptosis was analyzed by the oligonucleosome ELISA assay 24 hours after the start of the experiment. Results are expressed as a percentage of apoptotic cells of the total number of cultured cells. Data are the mean ± S.E.M. of 5 independent experiments. Significant difference vs. control: *p<0.05; **p<0.02, (Student's t test).

The results are shown in Figure 6 and show that PTX abolished the anti-apoptotic effect of guanosine. The number of apoptotic cells in PTX-treated astrocytes was not significantly different from that elicited by staurosporine alone. These results indicate that the protection by guanosine is mediated by a G_i-protein coupled plasma membrane binding site for guanosine, and this is most likely the putative guanosine receptor.

Guanosine-mediated phosphorylation of Akt/PkB in astrocytes

Guanosine-mediated phosphorylation of Akt (protein kinase B) in cultured rat astrocytes was evaluated by Western blot analysis. The results

-14 -

are shown in Figure 2. After 72 hours of re-plating, astrocytes were serum starved in high glucose for an additional 24 hours. Astrocytes were treated in panel A with control or with increasing concentrations of guanosine for 10 min. In panel B, astrocytes were pre-treated for 30 min with either the selective A1 adenosine receptor antagonist (DPCPX 100 nM) plus the selective A2B receptor antagonist (alloxazine 10 μ M) plus the non-selective P2 purine receptor antagonist (suramin 10 μ M), or with the PI-3 kinase inhibitor LY294002 (30 μ M) prior to the addition of 300 μ M guanosine. The antagonists were maintained also during the stimulation period (10 min) with guanosine.

10 Cell lysates (10 μ g) were resolved by SDS-PAGE and transferred to PVDF membranes before being probed with antisera specific for phosphorylated PKB (Ser⁴⁷³). The immunoblots are representative of 4 independent experiments. Blots were analysed by densitometric analyses and results are reported in the panels under each blot. In the panels A and B, the values are

15 given as means \pm S.E.M. of four experiments.

P* < 0.05, ** *P* < 0.01, ***** *P* < 0.001: significant difference versus control (Student's two-tailed t-test).

Figure 3 also shows guanosine-mediated phosphorylation of Akt/PKB in cultured rat astrocytes.

20 a) Effect of increasing concentrations of guanosine (Guo) on Akt/PKB phosphorylation.

Astrocytes were grown in medium containing 2% FBS for 24 hours, then were exposed to varying concentrations of guanosine (30, 100 or 300 μ M) for 10 minutes. At the end of the indicated treatment times astrocytes

25 were washed twice with ice-cold phosphate buffered saline (PBS) and harvested at 4°C. The expression of phosphorylated Akt/PKB was determined by Western immunoblot analysis as described in the Methods section. Immunoblots were quantitated by densitometric analysis, and reported in the panels below each blot. Data presented are representative of

30 4 independent experiments, with similar results. Maximal phosphorylation of Akt/PKB is detected following exposure of astrocyte cultures to 300 μ M guanosine.

-15 -

b) Time course of guanosine-stimulated Akt/PKB phosphorylation.

Experiments were performed as described for Figure 2, and astrocytes were exposed to 300µM guanosine (Guo) for varying times, and the expression of phosphorylated Akt/PKB was determined as in Figure 2. Maximal phosphorylation of Akt/PKB is detected after 5 minutes of guanosine exposure of astrocytes.

c) Effect of inhibitors of PI-3 kinase (LY 294002) and the combined antagonists of adenine-base purines (ABP) (DPCPX + DMPX + suramin) on the guanosine (Guo)-stimulated Akt/PKB phosphorylation.

Experiments were performed as described for Figure 2. Astrocytes were pre-treated for 30 minutes prior to the addition of guanosine with either the inhibitor of PI-3 kinase (30 µM LY 294002), or the combined antagonists of adenine-base purines (ABP) (100 nM DPCPX + 30 µM DMPX + 10 µM suramin), and the expression of phosphorylated Akt/PKB was determined as in Figure 2. Treatment of astrocytes with the PI-3 kinase inhibitor reduced guanosine-stimulated Akt/PKB phosphorylation to values below basal levels. Exposure of astrocytes to the combined antagonists of adenine-base purines (ABP) had no significant effect on guanosine-stimulated Akt/PKB phosphorylation.

Effect of guanosine on pro-apoptotic and cell survival pathways

In several cell types, staurosporine-induced apoptosis has been associated with the activation of multiple intracellular effectors and pathways, such as caspases, the efflux of cytochrome c from mitochondria (Deshmukh and Johnson, 2000; Belmokhtar et al., 2001), or the p38 MAP kinase pathway (Mirkes et al., 2000). The inventors therefore pre-treated astrocytes with the selective inhibitors of p38 MAP kinase (SB 202190, 1 µM), or caspases (z-VAD-fmk, 200 µM), and evaluated the effect of these agents on staurosporine-induced apoptosis. Administration of SB 202190, or z-VAD-fmk alone or in combination, reduced, but did not abolish the pro-apoptotic activity of staurosporine (by $68.2 \pm 4.2\%$ or $75.4 \pm 3.9\%$, respectively, and by $86.2 \pm 5.3\%$ when administered in combination) (Table 4). These data suggest, that

-16 -

the pro-apoptotic mechanisms of staurosporine in astrocytes involve p38 MAP kinase and caspase activation.

Pre-treatment of astrocytes with guanosine for 1 hour, prior to the addition of staurosporine reduced the number of apoptotic cells from $53.6 \pm 4.9 \%$ to $23.0 \pm 3.1 \%$; $p < 0.001$. When cells were pre-treated for 15 minutes with either SB 202190 or z-VAD-fmk alone or in combination, prior to the addition of guanosine, the number of apoptotic cells was again reduced (Table 4). These results therefore suggest that the anti-apoptotic effect of guanosine may be mediated, at least in part, by the p38 MAP kinase pathway and the caspase system.

To test whether other intracellular survival pathways are also involved in the anti-apoptotic activity of guanosine, the inventors pre-treated astrocytes with $30 \mu\text{M}$ LY 294002, a selective inhibitor of the PI 3-kinase prior to the administration of guanosine. The addition of LY 294002 had no significant effect on the apoptosis-promoting activity of staurosporine, whereas it completely abolished the anti-apoptotic effect of guanosine (Table 4). These data indicate that one of the main pathways activated by guanosine to reverse staurosporine-induced apoptosis of astrocytes includes the intracellular enzyme, PI 3-kinase.

Figure 1 is a bar graph showing the effect of guanosine on staurosporine-induced apoptosis and intracellular pathways. Effect of selected inhibitors of intracellular pathways on the percent of apoptotic astrocytes exposed to staurosporine in the absence or presence of guanosine. Astrocytes were exposed to guanosine ($300 \mu\text{M}$) for 1 hour before being exposed to staurosporine (100 nM) for 3 hours. At the end of this time period, the media was removed and replaced with DMEM without the pharmaceutical agents, and maintained for the entire duration of the experiment. When present, astrocytes were pre-treated for 30 minutes before the addition of guanosine with the following inhibitors: [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] (LY294002), an inhibitor of PI 3-kinase or [4-(4-fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole, FHPI] (SB202190), an inhibitor of the p38 pathway. Astrocyte apoptosis was

-17 -

analyzed at the 48h-time point by TUNEL assay as reported in the methods section. Apoptotic cells were counted and expressed as a percentage of the total cell number. Values are means \pm SEM of at least 4 independent experiments. Statistical significance was evaluated by Student's *t*-test;

5 ** P < 0.005 compared to staurosporine treatment alone.

 * P < 0.05 compared to staurosporine treatment alone.

Guanosine inhibits glycogen synthase kinase-3 β (GSK-3 β)

One of the physiological substrates of Akt/PKB in the cell survival pathway is GSK-3 β (Papp and Cooper, 1998; Hetman et al., 2000). The
10 inventors therefore evaluated whether treatment of cultured astrocytes with
guanosine had any effect on this intracellular target. Astrocytes, were grown
in culture medium containing 2% FBS for 24 hours, then were treated with
300 μ M guanosine (Guo) for the indicated time periods. Cell lysates (60 μ g of
protein per lane) were analysed by Western immunoblots using a specific
15 antibody against phospho- glycogen sythases kinase-3 (GSK-3 β , Ser9), the
isoform of GSK-3 β phosphorylated by the activation of the PI-3K/AKT
pathway. Immunoblots were quantified by densitometry as described in the
Methods section. Data presented are representative of at least three
independent experiments with similar results. Maximal phosphorylation of
20 GSK-3 β is detected after 5 minutes of guanosine exposure of astrocytes.

Exposure of cultured astrocytes to 300 mM guanosine for 30 min
increased phosphorylation of GSK-3 β at serine 9, in a time dependent
manner, inhibiting the pro-apoptotic activity of this enzyme. Phosphorylation
of GSK-3 β was rapid, and was maximal by 5 minutes following guanosine
25 addition, and declined to near baseline values after 30 minutes of guanosine
exposure (Figure 4). These results indicate that in cultured astrocytes the anti-
apoptotic effect of guanosine is mediated by inactivation of GSK-3 β , a
downstream target of the PI3K/Akt/ PKB pathway.

Guanosine induces Bcl-2 mRNA and protein expression

30 Another important downstream target of the PI3K/Akt/PKB cell survival
pathway is the Bcl-2 protein (Hengartner, 2000), which plays an important

-18 -

anti-apoptotic role. In the present study the inventors determined the expression of Bcl-2 mRNA and Bcl-2 protein in astrocytes, cultured in 2 % FBS containing medium and in cells following guanosine administration. Cultured rat astrocytes were pre-treated with guanosine (300 μ M) for 4 hours, and total RNA was isolated from untreated and guanosine-treated cells as described in the Methods section. RNA was transferred to membranes and hybridized with P^{32} -labelled Bcl-2 cDNA, synthesized and amplified as described in the Methods. Bcl-2 mRNA was analysed by gel electrophoresis on 1 % agarose gel, and normalized against GAPDH. The expression of Bcl-2 protein was detected by Western immunoblot analysis, and normalized against β -actin and the results are shown in Figure 5. The top right panel presents a representative Northern blot of Bcl-2 mRNA expression and the right panel a representative Western immunoblot of Bcl-2 protein concentration. The lower panel presents the results of densitometric analyses of the Northern blots of Bcl-2mRNA, normalized to GAPDH, and the Western blots of Bcl-2 protein, normalized to β -actin at 2, 4 and 6 hours after guanosine treatment. Data are means \pm S.E.M. of three independent experiments.

It was found that guanosine administration induced the expression of Bcl-2 mRNA 4 hours after exposure to this nucleoside, and it increased Bcl-2 protein expression 6 hours after guanosine exposure (Figure 5). These results confirm that in cultured astrocytes one of the intracellular targets mediating the protective effect of guanosine is the anti-apoptotic protein, Bcl-2.

Guanosine protects against apoptosis induced in astrocytes and neuron-like cells by exposure to deprivation of glucose and oxygen, a model of cerebral ischemia.

Guanosine protected against apoptotic death induced by the exposure of astrocytes and neuroblastoma cells to combined hypoxia-hypoglycemia (combined oxygen-glucose deprivation) (CGOD) for 1 hour. CGOD is an in vitro model of cerebral ischemia, as in stroke or cerebral hypoxia due to circulatory insufficiency.

-19 -

This kind of insult provokes a reduction of about 20 % in the number of total cells which remained attached to the dishes or glass coverslips after washing cells for further assays (fixation and staining by DAPI or MTS test), performed 24 h after cell exposure to 1 h CGOD. In astrocytes not exposed to CGOD (basal) the percentage of apoptotic cell was about 11%. This number increased to 27% when cells were submitted to 1 h CGOD. Guanosine (300 μ M) pre-treatment (started 1 h before CGOD and continued during 1 h of CGOD) protected the cells, being the number of apoptotic cells decreased to 11%. In neuroblastoma (SH-SY5Y) cells, the percentage of apoptotic cells in basal condition was about 8%; this number increased up to 18% as a consequence of cell exposure to 1 h CGOD. The pretreatment with guanosine (in the same conditions as described above) significantly decreased the proportion of apoptotic cells, to 12%.

DISCUSSION

Extracellular guanosine has a variety of trophic effects. It stimulates proliferation of astrocytes (Kim et al., 1991 and Ciccarelli et al., 2000) and other cells (Rathbone et al., 1992), stimulates the synthesis and release by astrocytes and microglia of neurotrophic and pleiotrophic factors such as NGF, S-100 β and TGF- β (Middlemiss et al., 1995; Caciagli et al., 2000; Ciccarelli et al., 1999; Di Iorio et al., 2001), exerts possible neurotrophic effects (Frizzo et al., 2001). Guanosine also enhances outgrowth of neuritic processes from neurons and neuron-like cells (Gysbers and Rathbone, 1992; Rathbone and Juurlink, 1993) and does not induce apoptosis in astrocytes (Di Iorio et al., 2001).

As the inventors have reported above, certain intracellular pathways are involved in the transduction signal of apoptosis. The PI 3-kinase/ Akt pathway is important in delivering an anti-apoptotic signal and the p38 mitogen-activated protein kinase pathway for promoting apoptosis. The inventors have demonstrated, that extracellular guanosine, alone, does not induce apoptosis (Di Iorio et al., 2002), however, when the PI 3-kinase pathway is inhibited (LY294002), guanosine does induce apoptosis in cultured rat astrocytes. To verify that extracellular guanosine does work through this

-20 -

particular pathway, the inventors have confirmed an increase in the phosphorylation of Akt by Western blot analysis and the production of the anti-apoptotic protein Bcl-2 by RT-PCR. In order to give credence to the hypothesis that extracellular guanosine is capable of rescuing/ protecting cells
5 from an apoptotic stimuli, the inventors pre-treated astrocytes with 300 μ M guanosine before the exposure of a known potent inducer of apoptosis, staurosporine, and found that this pre-treatment rescued 50% of the cell population from undergoing apoptosis. Finally the anti-apoptotic effect of guanosine was abolished by cell pre-treatment with LY294002.

10 To determine if the anti-apoptotic effect of guanosine was a phenomenon occurring only in astrocytes, the inventors examined the antiapoptotic effect of extracellular guanosine on differentiated SK-N-SH neuronal-type cells. The inventors found that once again, guanosine does not induce apoptosis in these types of cells. The inventors administered a
15 different inducer of apoptosis to correlate the anti-apoptotic effect of guanosine directly to Alzheimer's disease, where neurons are continually being lost due to apoptosis due to the accumulation of senile plaques caused by abnormal processing of amyloid precursor protein. In these cultures the inventors found that extracellular guanosine was able to once again rescue
20 50% of the cells from the β -amyloid apoptotic stimulus.

To determine whether this effect was limited to the non-adenine based purine nucleoside, guanosine, the inventors began testing other purine nucleosides such as inosine. The inventors found that inosine, like guanosine does not induce apoptosis in cultured rat astrocytes (Di Iorio et al., 2002) and
25 has neurorestorative effects (Benowitz et al., 1998, 1999). The inventors found that this nucleoside is also capable of protecting neuronal-type cells by more than 50%, from undergoing apoptosis.

While the present invention has been described with reference to what are presently considered to be the preferred examples, it is to be understood
30 that the invention is not limited to the disclosed examples. To the contrary, the invention is intended to cover various modifications and equivalent arrangements included within the spirit and scope of the appended claims.

-21 -

All publications, patents and patent applications are herein incorporated by reference in their entirety to the same extent as if each individual publication, patent or patent application was specifically and individually indicated to be incorporated by reference in its entirety.

-22 -

Table 1

Effect of guanosine (A. 100 μ M or B. 300 μ M) alone or in combination on the percent of β -amyloid induced apoptosis in differentiated neuronal SK-N-SH cells after 24 hours.

5

A.

Treatment	Average
Control	6 \pm 1.5
DMSO vehicle	10 \pm 3.5
NaOH vehicle	10 \pm 5.7
Staurosporine 10 nM	32 \pm 12
Guanosine 100 μ M	4 \pm 1.5
β -amyloid 5 μ M	21 \pm 7.8
Guanosine 100 μ M + β -amyloid 5 μ M	13 \pm 6.4
β -amyloid 10 μ M	28 \pm 8.9
Guanosine 100 μ M + β -amyloid 10 μ M	6 \pm 1.5

B.

Treatment	Average
Control	5.5 \pm 2.1
DMSO vehicle	11.5 \pm 4.9
NaOH vehicle	5 \pm 1.4
Staurosporine 10 nM	42 \pm 2.8
Guanosine 300 μ M	7 \pm 1.4
β -amyloid 5 μ M	16.5 \pm 0.7
Guanosine 300 μ M + β -amyloid 5 μ M	8.5 \pm 3.5
β -amyloid 10 μ M	25.5 \pm 9.2
Guanosine 300 μ M + β -amyloid 10 μ M	11.5 \pm 3.5

Cells were pre-treated with guanosine (100 μ M or 300 μ M) for 1 hour before exposure to β -amyloid. Cells were then exposed to (25-35) β -amyloid (5 μ M or 10 μ M) for the duration of the experiment. Exposure to guanosine and / or β -amyloid was maintained for the entire duration of the experiment. Apoptosis was analyzed at the 24-hour time point by staining with acridine orange/ethidium bromide as reported in the methods section. Apoptotic cells were counted and expressed as a percentage of the average of the total number of cells counted. Each average represents the mean \pm SD of (A) 3 independent experiments or (B) 2 independent experiments.

-23 -

Table 2

Effect of guanosine (A. 100 μ M or B. 300 μ M) alone or in combination on the percent of β -amyloid induced apoptosis in differentiated neuronal SK-N-SH cells after 48 hours of treatment.

5 A.

Treatment	Average
Control	4.7 \pm 1.2
DMSO vehicle	12 \pm 6.1
NaOH vehicle	11.3 \pm 7.6
Staurosporine 10 nM	41.3 \pm 16.2
Guanosine 100 μ M	7 \pm 3.6
β -amyloid 5 μ M	28.7 \pm 8.1
Guanosine 100 μ M + β -amyloid 5 μ M	12 \pm 5
β -amyloid 10 μ M	36.7 \pm 12.1
Guanosine 100 μ M + β -amyloid 10 μ M	15.3 \pm 6.4

B.

Treatment	Average
Control	3.5 \pm 2.1
DMSO vehicle	10.5 \pm 0.7
NaOH vehicle	16 \pm 2.8
Staurosporine 10 nM	42 \pm 8.5
Guanosine 300 μ M	12.5 \pm 3.5
β -amyloid 5 μ M	33.5 \pm 0.7
Guanosine 300 μ M + β -amyloid 5 μ M	11 \pm 1.4
β -amyloid 10 μ M	48 \pm 9.9
Guanosine 300 μ M + β -amyloid 10 μ M	18 \pm 2.8

Cells were pre-treated with guanosine (100 μ M or 300 μ M) for 1 hour before exposure to β -amyloid. Cells were then exposed to (25-35) β -amyloid (5 μ M or 10 μ M) for the duration of the experiment. Exposure to guanosine and/or β -amyloid was maintained for the entire duration of the experiment. Apoptosis was analyzed at the 24-hour time point by staining with acridine orange/ethidium bromide as reported in the methods section. Apoptotic cells were counted and expressed as a percentage of the average of the total number of cells counted. Each average represents the mean \pm SD of (A) 3 independent experiments or (B) 2 independent experiments.

-24 -

Table 3

Effect of inosine 100 μ M alone or in combination on the percent of β -amyloid induced apoptosis in differentiated neuronal SK-N-SH cells after 24 (A) or 48 hours (B) following treatment.

5

A.

Treatment	Average
Control	9
DMSO vehicle	8
Staurosporine 10 nM	26
Inosine 100 μ M	6
β -amyloid 5 μ M	25
Inosine 100 μ M + β -amyloid 5 μ M	11
β -amyloid 10 μ M	25
Inosine 100 μ M + β -amyloid 10 μ M	17

B.

Treatment	Average
Control	7
DMSO vehicle	6
Staurosporine 10 nM	32.5
Inosine 100 μ M	3
β -amyloid 5 μ M	25
Inosine 100 μ M + β -amyloid 5 μ M	10.5
β -amyloid 10 μ M	20.5
Inosine 100 μ M + β -amyloid 10 μ M	12.5

10

Cells were pre-treated with inosine (100 μ M) for 1 hour before exposure to β -amyloid. Cells were then exposed to (25-35) β -amyloid (5 μ M or 10 μ M) for the duration of the experiment. Exposure to inosine and/or β -amyloid was maintained for the entire duration of the experiment. Apoptosis was analyzed at the 24-hour time point (A) of 48-hour time point (B) by staining with acridine orange/ethidium bromide as reported in the methods section. Apoptotic cells were counted and expressed as a percentage of the average of the total cell number counted.

15

Table 4. Effect of selected inhibitors of intracellular pathways on apoptosis induced by staurosporine in rat brain cultured astrocytes in the absence and in the presence of guanosine

5

	<u>No Guano sine</u>		<u>Guanosine 300 μM</u>	
	Basal	Staurosporine 100 nM	Basal	Staurosporine 100 nM
<u>Control</u>	5.4 \pm 0.7 (n = 5)	53.6 \pm 4.9 (n = 6) §	1.9 \pm 0.5 (n = 6)	23.0 \pm 3.1 (n = 6) #
<u>SB202190 1 μM</u>	4.9 \pm 1.1 (n = 5)	17.1 \pm 2.6 (n = 5) ***	2.8 \pm 0.4 (n = 5)	16.3 \pm 1.7 (n = 5) *
<u>z-VAD-fmk 200 μM</u>	4.4 \pm 0.8 (n = 5)	13.2 \pm 1.9 (n = 5) ***	2.4 \pm 0.2 (n = 5)	15.2 \pm 1.6 (n = 5) *
<u>SB202190+z-VAD-fmk</u>	4.1 \pm 0.5 (n = 5)	7.7 \pm 1.7 (n = 5) ***	2.3 \pm 0.5 (n = 5)	10.4 \pm 2.2 (n = 5) **
<u>LY294002 30 μM</u>	6.1 \pm 1.2 (n = 6)	59.2 \pm 5.9 (n = 6)	7.1 \pm 1.3 (n = 6) **	57.9 \pm 6.8 (n = 6)

Cells were exposed for 3 h to staurosporine (100 nM) in a DMEM with 2% FCS to induce apoptosis (positive control). Guanosine (300 μ M) was added to the culture medium 1 h before staurosporine exposure. When indicated, cell pretreatments with [4-(4-(fluorophenyl)-2-(4-hydroxyphenyl)-5-(4-pyridyl)1H-imidazole, FHPI] (SB202190) or [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one] (LY294002) or z-VAD-fmk were carried out starting from 30 min before astrocyte exposure to guanosine and/or staurosporine and maintained for the entire duration of the experiment. Astrocyte apoptosis was analysed at the 24 h time point by acridine orange/ethidium bromide staining as reported in the method section. Apoptotic cells were counted as a percentage of the total cell number. Values are mean \pm SEM of 5-6 independent experiments. Statistical significance was evaluated by Student's t-test; § P < 0.001 compared with the respective basal value; * p < 0.05 ** p < 0.01 *** p < 0.001 compared with respective positive control; # p < 0.001 compared with positive control in the absence of guanosine.

FULL CITATIONS FOR REFERENCES REFERRED TO IN THE SPECIFICATION

- 5 Belmokhtar CA, Hillion ZJ, Ségal-Bendirdjian E. 2001. Staurosporine induces apoptosis through both caspase-dependent and caspase-independent mechanisms. *Oncogene*, 20:3354-3362.
- 10 Benowitz LI, Jing Y, Tabibiazar R, Jo SA, Petrusch B, Struemer CA, Rosenberg PA, Irwin N. 1998. Axon outgrowth is regulated by an intracellular purine-sensitive mechanism in retinal ganglion cells. *J Biochem* 273(45):29626-34.
- 15 Benowitz LI, Goldberg DE, Madsen JR, Soni D, Irwin N. 1999. Inosine stimulates extensive axon collateral growth in rat corticospinal tract after injury. *Proc Natl Acad Sci U.S.A.* 96(23):13486-90.
- 20 Caciagli F, Di Iorio P, Guilianni P, Middlemiss PJ, Rathbone MP. 2000. The neuroprotective activity of guanosine involves the production of trophic factors and the outflow of purines from astrocytes. *Drug dev Res Abstract* 50 (1):32.
- 25 Ciccarelli R, Di Iorio P, D'alimonte I, Kleywegt S, Ballerini P, Rathbone MP, Poli A Caciagli F. 1999. Guanosine and related drugs stimulate the production of neurotrophic factors from rat cultured astrocytes by involving the mitogen-activated protein kinase pathway. *Neurosci Soc abstract* (29th annual Meeting) 25:1013:407.
- 30 Ciccarelli R, Di Iorio P, D'Alimonte I, Julianne P, Flora T, middlemiss PJ, Rathbone MP. 2000. Cultured astrocyte proliferation induced by extracellular guanosine involves endogenous adenosine and is raised by the co-presence of microglia. *Glia* 29:202-211.

Desmukh M, Johnson EM Jr. 2000. Staurosporine-induced neuronal death: multiple mechanisms and methodological implications. *Cell Death Differ* 7:250-261.

5

Di Iorio P, Caciagli F, Guilianni P, Ballerini P, Ciccarelli R, Sperling O, Zoref-Shani E, Benowitz L, Traversa U, Bombi G, Florio T, Virgilio A, Andrew CM, Crocker CE, Werstiuk ES, Middlemiss PJ, Rathbone MP, D'Alimonte I, Kleywegt S. 2001. Purine nucleosides protect injured neurons and stimulate neuronal regeneration by intracellular and membrane receptor-mediated mechanisms. *Drug Dev Res* 52:303-315.

10

Frizzo MES, Lara DR, Dahm KCS, Prokopiuk AS, Swanson RA, Souza DO. 2001. Activation of glutamate uptake by guanosine in primary astrocyte cultures. *Neuroreport* 12(4):879-81.

15

Gysbers JW and Rathbone MP. 1992. Guanosine enhances NGF-stimulated neurite outgrowth in PC12 cells. *Neuroreport* 3:997-1000.

20 Hengartner MO. 2000. The biochemistry of apoptosis. *Nature* 407: 770-776.

Kennedy SG, Wagner AJ, Conzen SD, Jordan J, Bellacosa A, Tsichlis PN, Hay N. 1997. The PI 3-kinase/ Akt signaling pathway delivers an anti-apoptotic signal. *Genes and Dev* 11: 701-713.

25

Kim HA, Khursigara G, Sun X, Franke TF, Chao MV. 2001. Akt phosphorylates and negatively regulates apoptosis signal-regulating kinase 1. *Mol Cell Bio* 21(3): 893-901.

30 Kim JK, Rathbone MP, Middlemiss P, Hughes DW, Smith R. 1991. Purinergic stimulation of astroblast proliferation: guanosine and its nucleotides stimulate cell division in chick astroblasts. *J Neurosci Res* 28:442-455.

- McConkey DJ and Orreius S. 1994. Signal transduction pathways. Trends Cell Biol 4:370-374.
- 5 Middlemiss PJ, Gysbers JW, Rathbone MP. 1995. Extracellular guanosine and guanosine-5'-triphosphate increase NGF synthesis and release from cultured mouse neopallial astrocytes. Brain Res 677:152-156.
- Mirkes PE, Wison KL, Cornel LM. 2000. Teratogen-induced activation of ERK,
10 JNK, and p38 MAP kinases in early postimplantation murine embryos. Teratology 62:1 4-25.
- Orike N, Middleton G, Borthwick E, Buchman V, Cowen T, Davies AM. 2001. Role of PI 3-kinase, Akt and Bcl-2-related proteins in sustaining the
15 survival of neurotrophic factor-independent adult sympathetic neurons. J Cell Bio 154(5):995-1005.
- Pap M, Cooper GM. 1998. Role of glycogen synthase kinase-3 in the phosphatidylinositol 3-kinase/Akt cell survival pathway. J Biol Chem
20 273:19929-19932.
- Preis PN, Saya H, Nadasdi L, Hochhaus G, Levin V, Sadee W. 1998. Neuronal cell differentiation of human neuroblastoma cells by retinoic acid plus herbimycin A. Cancer Res 48:6530-6534.
25
- Rathbone MP, Middlemiss PJ, Gysbers JW, Deforge S, Costello P, Del Maestro RF. 1992. Purine nucleosides and nucleotides stimulate proliferation of a wide variety of cell types. In vitro Cell Dev Biol 28A:529-536.
30
- Rathbone MP and Juurlink B. 1993. Hippocampal neurons in vitro respond to extracellular guanosine and GTP with neurite outgrowth and branching.

-29 -

Soc. Neurosci 23rd annual meeting, Washington, DC, Nov 7-12, 19:38
abstract 4.7.

Traversa U, Bombi G, Di Iorio P, Ciccarelli R, Werstiuk ES, Rathbone MP.
5 (2002a) Specific [^3H]-guanosine binding sites in rat brain membranes. Br J
Pharmacol 135: 969-76.

Traversa U., Di Iorio P., Palmieri C., Bombi G., Ciccarelli R., Caciagli F.
10 (2002b) Identification of a guanosine receptor linked to the modulation of
adenylate cyclase and MAPK activity in primary cultures of rat astrocytes.
Proceedings of the Annual Meeting of Purine Club, Bertinoro, Italy,
October 27-28, 2002, p. 9.

Wang E, Marcotte R, Petroulakis E. 1999. Signaling Pathway for Apoptosis; a
15 racetrack for life or death. J Cell biochem Suppl 32/33:95-102.

Wang E, Marcotte R, Petroulakis E. 1999. Signaling Pathway for apoptosis; a
racetrack for life or death. J Cell Biochem Suppl 32/33:95-102.

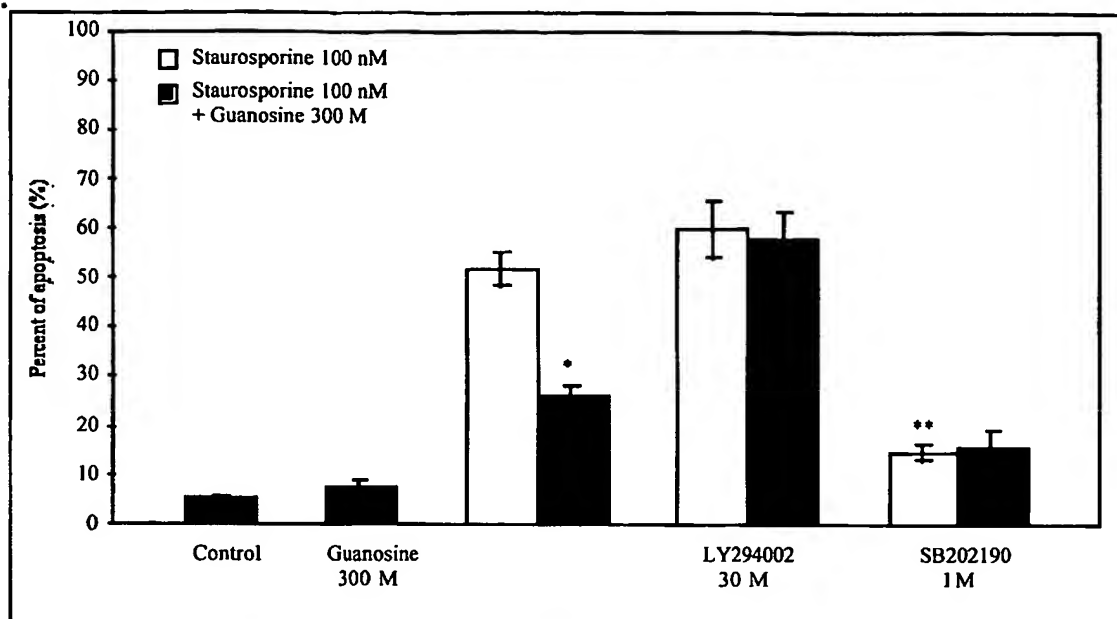
-30 -

WE CLAIM:

1. A use of an effective amount of a purine nucleoside selected from guanosine, inosine or an analog of guanosine or inosine for the manufacture
5 of a medicament for preventing apoptosis in a cell of the nervous system.
2. The use according to claim 1 wherein the cell of the nervous system is from the central nervous system.
- 10 3. The use according to claim 2 wherein the cell is an astrocyte or a neuron.
4. The use according to any one of claims 1 to 4 for the manufacture of a medicament for preventing or treating a neurodegenerative disease, a stroke,
15 a head injury or a spinal cord injury.
5. The use according to claim 4 wherein the neurodegenerative disease is selected from Alzheimer's disease, Huntington's chorea or Parkinson's disease, Bell's Palsy, Pick's disease, multiple sclerosis and amyotrophic
20 lateral sclerosis, and prion-induced diseases.
6. The use according to claim 5 wherein the prion-induced disease is selected from Creutzfeldt-Jacob disease, scrapie, and bovine spongiform encephalopathy.
25
7. A pharmaceutical composition for preventing apoptosis in a cell of the nervous system comprising an effective amount of a purine nucleoside selected from guanosine, inosine or an analog of guanosine or inosine in admixture with a suitable diluent or carrier.
30
8. The pharmaceutical composition according to claim 7 wherein the cell of the nervous system is from the central nervous system.

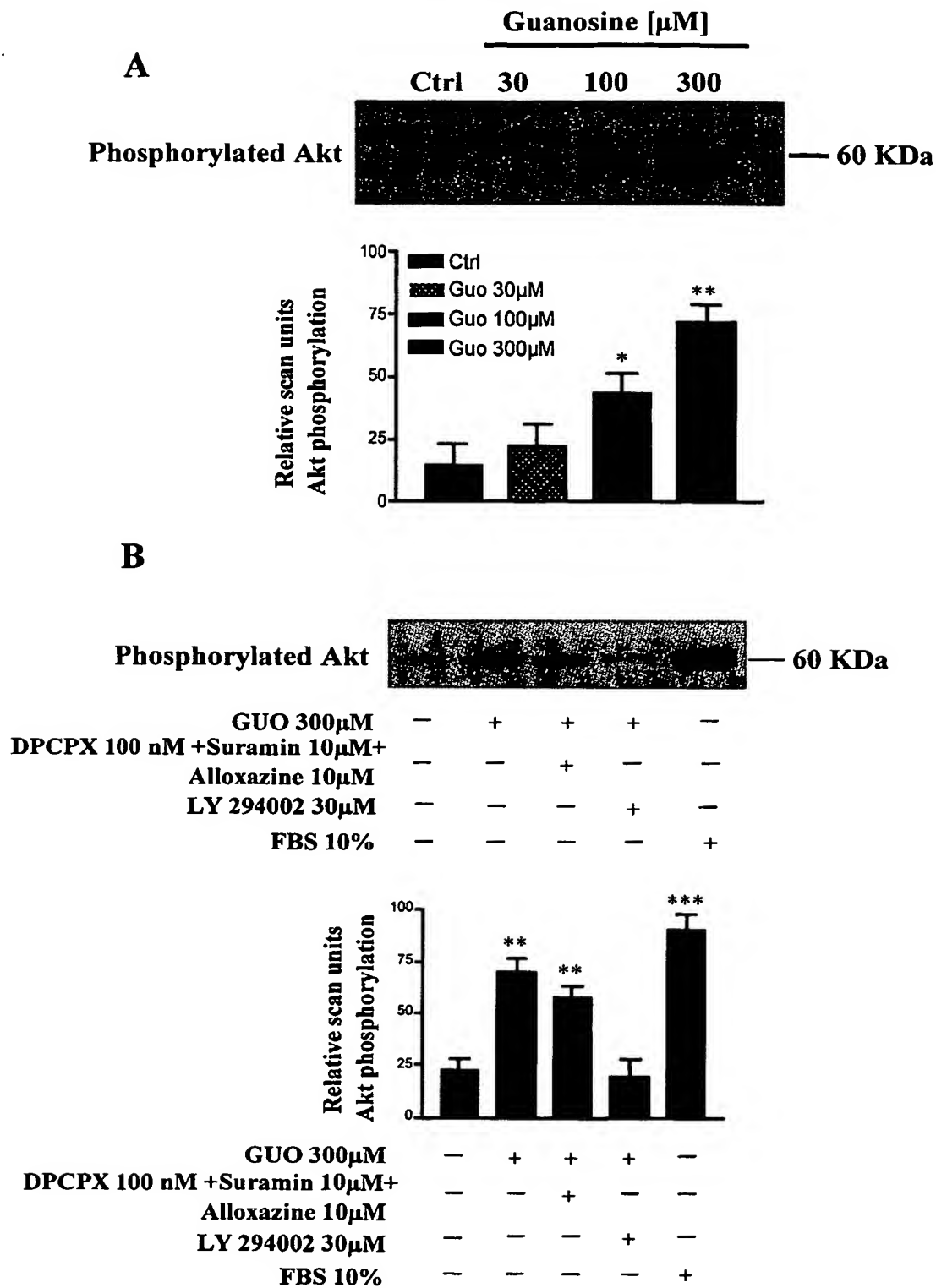
9. The pharmaceutical composition according to claim 7 wherein the cell is an astrocyte or a neuron.
- 5 10. The pharmaceutical composition according to any one of claims 7 to 9 for the manufacture of a medicament for preventing or treating a neurodegenerative disease, a stroke, a head injury or a spinal cord injury.
- 10 11. The pharmaceutical composition according to claim 10 wherein the neurodegenerative disease is selected from Alzheimer's disease, Huntington's chorea or Parkinson's disease, Bell's Palsy, Pick's disease, multiple sclerosis and amyotrophic lateral sclerosis, and prion-induced diseases.
- 15 12. The pharmaceutical composition according to claim 11 wherein the prion-induced disease is selected from Creutzfeldt-Jacob disease, scrapie, and bovine spongiform encephalopathy.
- 20 13. A use of an effective amount of a purine nucleoside selected from guanosine, inosine or an analog of guanosine or inosine for the manufacture of a medicament for preventing glycogen synthase kinase-3 β activity in a cell of the nervous system.
- 25 14. A use of an effective amount of a purine nucleoside selected from guanosine, inosine or an analog of guanosine or inosine for inducing the expression and/or activity of Bcl-2 in a cell of the nervous system.

1/6

FIGURE 1

2/6

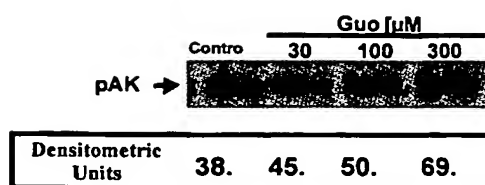
FIGURE 2



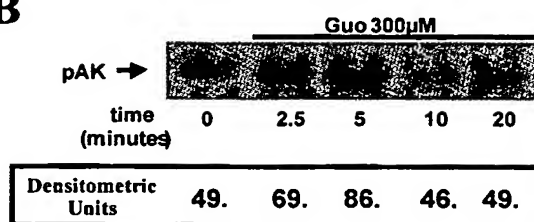
3/6

FIGURE 3

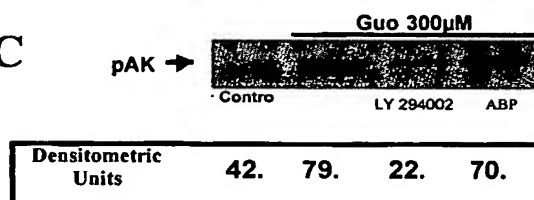
A



B

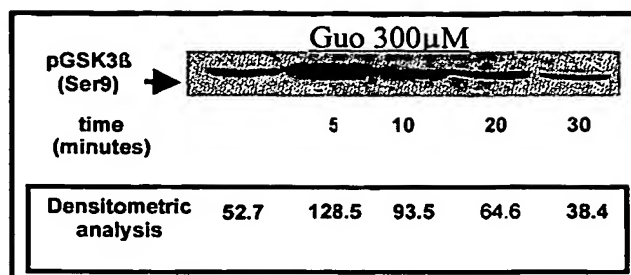


C



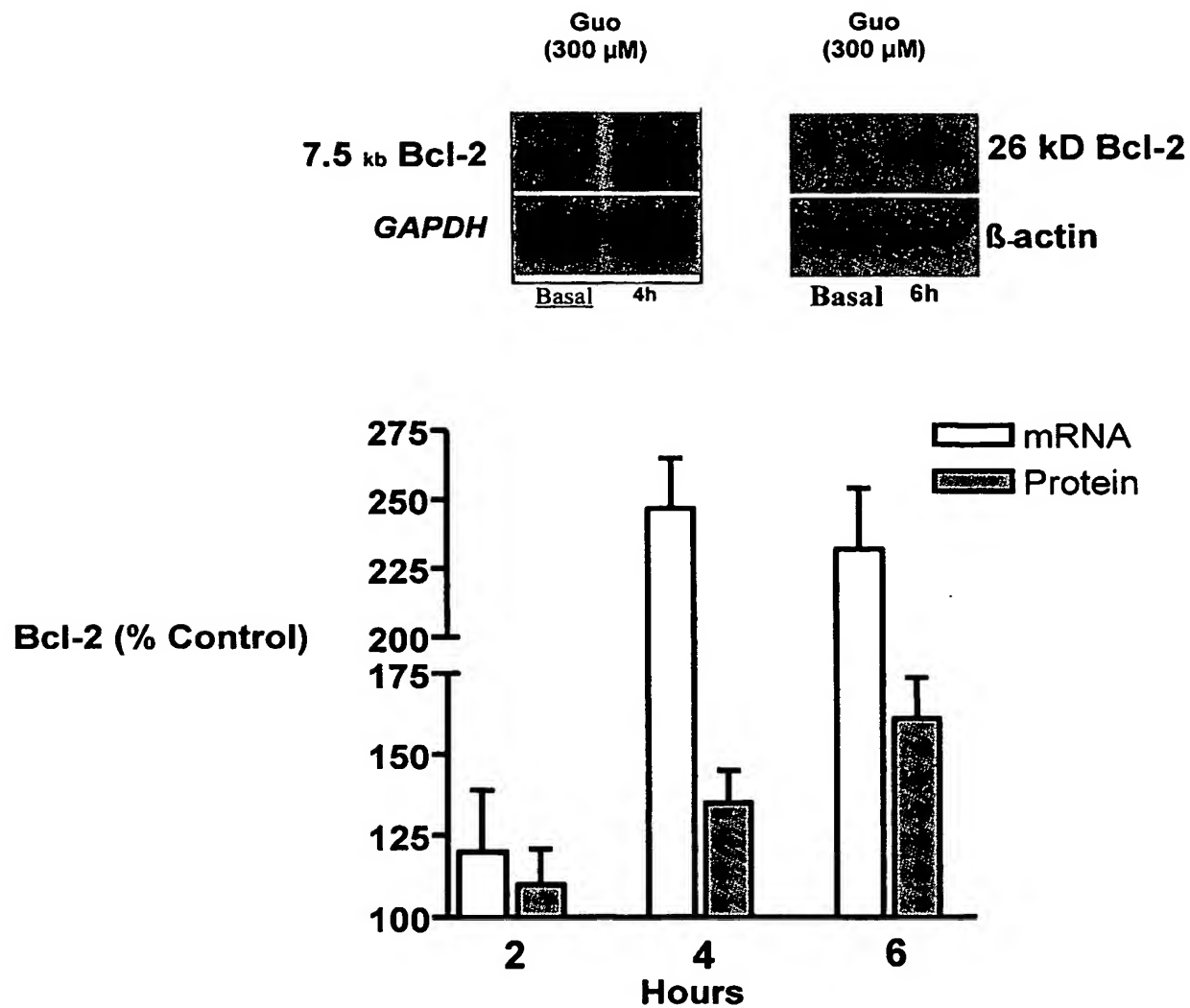
4/6

FIGURE 4

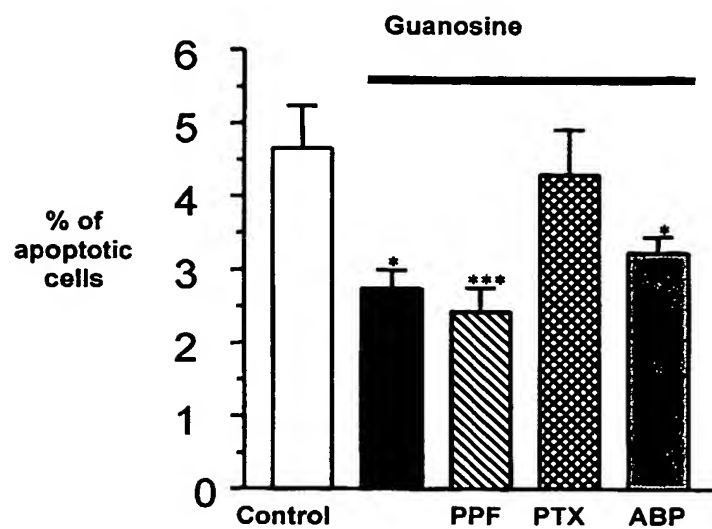


5/6

FIGURE 5



6/6

FIGURE 6

**This Page is Inserted by IFW Indexing and Scanning
Operations and is not part of the Official Record**

BEST AVAILABLE IMAGES

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images include but are not limited to the items checked:

- ☐ BLACK BORDERS
- ☐ IMAGE CUT OFF AT TOP, BOTTOM OR SIDES
- ☒ FADED TEXT OR DRAWING
- ☒ BLURRED OR ILLEGIBLE TEXT OR DRAWING
- ☐ SKEWED/SLANTED IMAGES
- ☐ COLOR OR BLACK AND WHITE PHOTOGRAPHS
- ☐ GRAY SCALE DOCUMENTS
- ☒ LINES OR MARKS ON ORIGINAL DOCUMENT
- ☒ REFERENCE(S) OR EXHIBIT(S) SUBMITTED ARE POOR QUALITY
- ☐ OTHER: _____

IMAGES ARE BEST AVAILABLE COPY.

As rescanning these documents will not correct the image problems checked, please do not report these problems to the IFW Image Problem Mailbox.